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Occurring Motoneuron Death Reflects the Time of Prior Exit from the Cell Cycle and Position within the Lateral Motor Column

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Embryonic lumbar spinal motoneurons (MNs) are characterized by a period of programmed cell death (PCD) that spans several days and occurs in a rostrocaudal gradient. The generation of these MNs also takes place in a temporal–spatial gradient, such that MNs within rostral lumbar segments exit the cell cycle earlier and MNs within progressively caudal regions are born later. *In vitro* studies have shown that the latest born spinal MNs, presumably through the possession of endogenous "survival properties," are also the last to acquire their trophic dependence. If the birth date and therefore spinal cord location of lumbar spinal MNs influence the spatial–temporal pattern of PCD, then earlier born MNs should die sooner and be located more rostrally than those generated later. Alternatively, if the time at which MNs die during development is unrelated to their prior exit from the cell cycle, those born at various phases should die throughout the period of PCD. We report here that lumbar MNs generated during the earliest part (embryonic day 2–3) of the proliferative period in the developing chick spinal cord tend to die during the earliest stages of the PCD period and that MNs born in successive 12-h intervals die at correspondingly later periods during PCD. Furthermore, the spatial progression of PCD of these subpopulations of MNs occurs in a rostrocaudal gradient. Finally, while MNs do appear to die in a mediolateral gradient during the period of MN PCD, this pattern is only partly accounted for by MNs born in consecutive intervals. These data support the notion that the timing and rostrocaudal location of MNs undergoing PCD reflect their time of exit from the cell cycle.

Key Words: **motoneuron; cell death; apoptosis; BrdU; spinal cord; birth date; cell cycle.**

INTRODUCTION

Developing spinal motoneurons (MNs) are characterized by specific regional and temporal differences in cell cycle exit, gene expression, cell number, target innervation, trophic factor dependency, and cell death pattern (Hollyday and Hamburger, 1977; Landmesser, 1978b; Oppenheim *et al.*, 1989; O'Brien and Oppenheim, 1990; Mettling *et al.*, 1993; Tsuchida *et al.*, 1994). The extent to which these qualities instruct or reflect the identity of specific subpopulations of MNs is a topic of intense research. For example, a null mutation of the *hoxc-8* gene alters the settling pattern of a subset of MNs within the spinal cord, potentiates cell death within this group of MNs, and results in abnormal neuromuscular function (Tiret *et al.*, 1998).

Regional diversification of MNs is represented by their classification into specific subpopulations. Four major rostrocaudal columns of somatic spinal MNs are defined by the type of musculature they innervate (Landmesser, 1978a; Hollyday, 1980a; Gutman *et al.*, 1993) and by the precise combination of LIM-homeodomain factors they express prior to innervation (Tsuchida *et al.*, 1994). Specific pools of MNs within these columnar classes are further defined by the individual muscle they innervate (Landmesser, 1978a,b) and by specific patterns of ETS transcription factor expression (Lin *et al.*, 1998). Spinal MNs are also characterized by region-specific variations in number, which first arise through differential proliferation (Oppenheim *et al.*, 1989) and are subsequently refined by the process of programmed cell death (PCD) (Hamburger, 1975). Avian spinal MNs

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become postmitotic between stage 14 and stage 23 (E2–E4.5) in a rostrocaudal gradient, such that the first wing-innervating brachial MNs are born at stage 15 and the first leg-innervating lumbar MNs are born at stage 17 (Hollyday and Hamburger, 1977). In addition, lumbar MNs exhibit a rostrocaudal gradient of PCD, which is complete by E9 in the rostral four lumbar segments but not until E12 in the caudal four segments (Williams *et al.*, 1987).

MNs are increasingly classified by their ability to respond to specific trophic factors. That these factors, including cardiotrophin-1, glial cell line-derived neurotrophic factor (GDNF), and hepatocyte growth factor (HGF), rescue only limited numbers of MNs suggests the existence of specific subpopulations of MNs defined by distinct trophic dependencies (Henderson *et al.*, 1994; Pennica *et al.*, 1996; Yamamoto *et al.*, 1997; Novak *et al.*, 1999). This hypothesis is also supported by the finding that *in ovo* administration of brain-derived neurotrophic factor prevents the PCD of lumbar MNs between E8 and E10 but not E6 and E8 (McKay *et al.*, 1996), by the demonstration in the chick that only lumbar MNs express the c-Met receptor for HGF during the period of PCD (Novak *et al.*, 1999) and by the discovery that the signal-transducing component of the GDNF receptor, c-Ret, is expressed within subpopulations of lumbar MNs (S. Homma and R. Oppenheim, unpublished observations).

The determination of such region-specific differences between these various classes of MNs has been investigated in the chick embryo by reversing or exchanging various portions of the early neural tube and assessing the change in MN identity, number, projection pattern, or cell death. For example, when three or four segments of the lumbar neural tube are reversed at stage 15 (50–55 h), innervation patterns are changed accordingly, but when reversed two stages earlier, projection patterns are normal (Lance-Jones and Landmesser, 1980; Matise and Lance-Jones, 1996). Transplantation of the thoracic to the lumbar neural tube at stage 14 (50–53 h) reduces the number of dying thoracic MNs, presumably by increasing the size of the target (O'Brien and Oppenheim, 1990), although MNs appear to be specified to innervate limb versus trunk muscles by stage 12 (45–49 h) (Tanaka *et al.*, 1997). Finally, reversal of thoracic and brachial neural tube segments at stage 11–12 (40–49 h) accordingly changes the number of region-specific MNs generated before PCD and changes their identity as defined by both LIM expression and Hoxc-8 expression (Ensini *et al.*, 1998). These studies suggest that regional differences between MNs are not specified by E2, but begin to appear shortly thereafter.

Because presumptive spinal MNs appear to be specified before or at the time of their birth, we sought to determine if birth date influences the temporal pattern of cell death of lumbar MNs. Data generated from *in vitro* studies suggest that spinal MNs acquire trophic factor dependence at a set time after their birth (Mettling *et al.*, 1995), perhaps as a reflection of intrinsic "survival properties" (Mettling *et al.*, 1993). We hypothesized that if extrinsic cues exclusively are sufficient to regulate the survival of MNs, then the time of birth should not influence the temporal pattern of death. According to this scheme, MNs born at different times should exhibit the same pattern of cell death between E6 and E10. If, however, MNs have an intrinsic clock which limits their survival time in the absence of trophic support, then earlier born MNs might be expected to die during early periods of MN PCD and later born MNs at a later time. Although these two possibilities (intrinsic vs extrinsic) are not necessarily mutually exclusive, the present experiments represent a beginning attempt to address their relative merits. Some of the results presented herein were previously reported in abstract form (Burek *et al.*, 1996).

MATERIALS AND METHODS

Administration of BrdU and Tissue Preparation

Fertilized chicken eggs were obtained from Tyson Farms (Wilkesboro, NC) and incubated at 37°C and 60% relative humidity until they reached the appropriate age for experimental manipulation. All eggs were staged according to the Hamburger and Hamilton stage series (1951), candled, and windowed at E2, which is close to the beginning of motoneuron genesis (Hollyday and Hamburger, 1977). The S-phase marker 5-bromo-2′deoxyuridine (BrdU; 2.5 μg (163 μM); Sigma) in a volume of 50 μl of 0.9% saline was administered topically to the vitelline membrane at E2.5, E3, E3.5, or E4. In some embryos, 15 μCi [^{3}H]thymidine (Amersham) in a volume of 50 μl saline was administered at specific times after delivery of BrdU (see below). Embryos were sacrificed during MN PCD at E6.5, E7.5, E9, and E10, and their spinal columns were fixed overnight at room temperature in Carnoy's solution. Spinal columns were subsequently dehydrated, embedded in paraffin, and sectioned serially on a rotary microtome at 6 (E6.5), 7 (E7.5), or 8 μm (E9 and E10). Alternate sections were placed on slides and stained with the nucleophilic dye thionin to identify the lumbar lateral motor column (LMC).

BrdU Immunohistochemistry and [^{3}H]Thymidine Autoradiography

Alternate tissue sections within the lumbar spinal cord were placed on slides, deparaffinized, incubated in 3% H_2O_2 to block endogenous peroxidase activity, and treated with 2 N HCl for 30 min at 37°C to denature double-stranded DNA. The sections were then treated with a 1:20 concentration of mouse monoclonal anti-BrdU antibody in 1% NGS/PBS (Becton–Dickinson), 1:100 HRP-conjugated goat anti-mouse IgG in 1% NGS/PBS (The Jackson Laboratory), and finally exposed to the peroxidase substrate diaminobenzidine (Vector), a chromophore whose nickel-enhanced reaction product permits the visualization of BrdU-labeled MNs. Healthy MNs were counted only if they exhibited at least one clear nucleolus and a continuous nuclear membrane (Clarke and Oppenheim, 1995). Dying MNs were identified by their characteristic pyknotic morphology, which includes a condensed and circular nucleus sometimes accompanied by apoptotic bodies (Clarke and Oppenheim, 1995). Healthy and pyknotic BrdU-labeled MNs were counted in every 10th section by a blinded observer, and the counts were multiplied by 10 to estimate total cell number.

In order to determine the duration of time after delivery that BrdU was still available to proliferating MNs, [^{3}H]thymidine was

TABLE 1

The Number (Mean ± SD per Section) of BrdU-Labeled, [^{3}H]Thymidine-Labeled, and BrdU/[^{3}H]Thymidine-Colabeled MNs in the Lumbar LMC at E13 after a Dose of BrdU at E2.5 and a Dose of [^{3}H]Thymidine 7, 14, 22, or 30 h Later

Interval (h) between the delivery of BrdU and [^{3}H]thymidine	BrdU-labeled MNs ± SD	[^{3}H]Thymidine-labeled MNs ± SD	Colabeled MNs ± SD
7	23 ± 2[a]	18 ± 2	16 ± 3
14	21 ± 4	15 ± 2	3 ± 1
22	28 ± 2	6 ± 12	0 ± 0
30	24 ± 4	3 ± 1	0 ± 0

[a] Values represent the average of five different sections from each of $n = 3$ embryos.

given 7, 14, 22, and 30 h after BrdU delivery at E2.5. Although the rate of tracer dilution for an individual cell depends upon the interval between delivery of the tracer and the onset of mitosis, we feel our method accurately measures the duration of BrdU availability based on the fact that the overall number of MNs labeled with BrdU *or* [^{3}H]thymidine at E13 was similar in each of these four groups (Table 1). In addition, summation of the number of BrdU-labeled MNs in each of the four groups (BrdU given at E2.5, E3, E3.5, and E4) at any time point during PCD (E6.5, E7.5, E9, or E10) results in MN numbers that exceed the published values for that time point by only ~20% (see Results and Discussion). Embryos were sacrificed at E13 and sectioned at 12 μm. After being immunostained for BrdU, these sections were rinsed in water; allowed to air dry; dipped in a 1:1 solution of Kodak NTB2 emulsion:0.1% Triton X; exposed for 2 weeks in light-tight boxes at 4°C, developed in 2.5% sodium sulfite, 0.1% potassium bromide, 0.45% 2,4 diaminophenol dihydrochloride and fixed in 5% sodium thiosulfate (Belecky-Adams *et al.*, 1996). [^{3}H]Thymidine-labeled MNs were identified by the same criteria used to include BrdU-labeled MNs described above. Cells with at least 25 silver grains in their nucleus, a density which significantly distinguishes labeled from unlabeled MNs in previous reports (Nornes and Das, 1974), were considered to be positively labeled.

RESULTS

BrdU Is Not Cytotoxic in Ovo

Because *in vivo* administration of the drug BrdU has been reported to suppress neurogenesis, to cause morphological defects, and to inhibit interdigital PCD (Hunt, 1975; Tone *et al.*, 1983; Sechrist and Bronner-Fraser, 1991), it was necessary to characterize the cytotoxicity of different dosages of BrdU. At a dose of 50 μg/egg, BrdU significantly reduced (12% vs 100% for controls) the survival of embryos when given at E2, E2.5, and E3 (data not shown). This is in agreement with a previous report which showed that 20 μg/egg BrdU delivered at E3 results in significant embryo mortality (Bannigan *et al.*, 1981). At 5 μg/egg, viability was 100% in embryos treated with BrdU at E3, E3.5, and E4, but only 50–60% in embryos treated at E2.5. Lowering the dose to 2.5 μg/egg resulted in 100% viability of these E2.5-treated embryos but still killed half the embryos given BrdU at E2. Of those E2-treated embryos which survived, most showed a marked spinal cord necrosis at E6. Therefore MNs born between E2 and E2.5, at the very earliest stages of spinal MN proliferation, were excluded from the study. Embryos which received BrdU exhibited an initial 6- to 18-h delay in development up until E6–E7, at which time their expected stage (Hamburger and Hamilton, 1951) and embryonic age had equilibrated. Of those embryos treated with BrdU at E2.5, MNs were counted both before (E6) and after (E13) the period of PCD and compared with controls (Fig. 1). No differences were found, suggesting that BrdU at this dose does not alter terminal MN differentiation or PCD and is not cytotoxic. Additionally, embryos given this dose of BrdU on E2.5 and examined 1–4 days later did not exhibit increased numbers of pyknotic MNs or sensory neurons within the lumbar LMC or the L3 DRG, respectively (data not shown). Finally, the number of healthy BrdU-labeled MNs at E7.5 was similar in embryos treated with either 2.5 or 25 μg/egg at E3.5 (data not shown), suggesting that this lower dose is of sufficient concentration to label all proliferating spinal MNs within a specific 12-h period.

BrdU Is Available to Proliferating MNs between 14 and 22 h after Delivery

In order to demonstrate that subpopulations of MNs could be reliably distinguished by adding BrdU at 12-h intervals, we assessed the length of time BrdU remained available for uptake by proliferating MNs. Previous studies report that

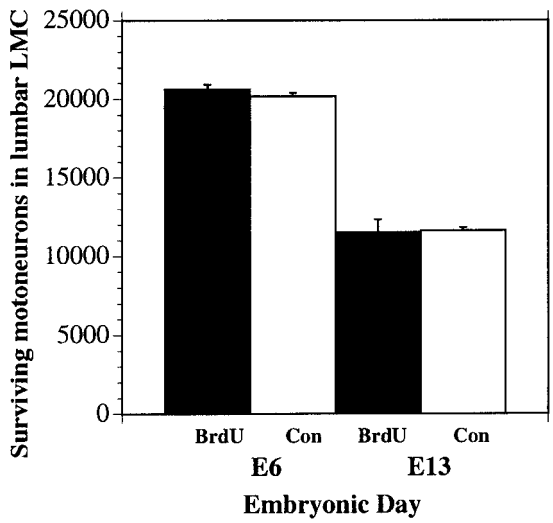


FIG. 1. BrdU treatment does not affect motoneuron (MN) proliferation or programmed cell death (PCD). The number (mean ± SD) of lumbar lateral motor column (LMC) MNs at E6, before the onset of MN PCD, and at E12, after the end of PCD, in both controls and in embryos given 2.5 μg BrdU at E2.5 is shown ($n = 3$).

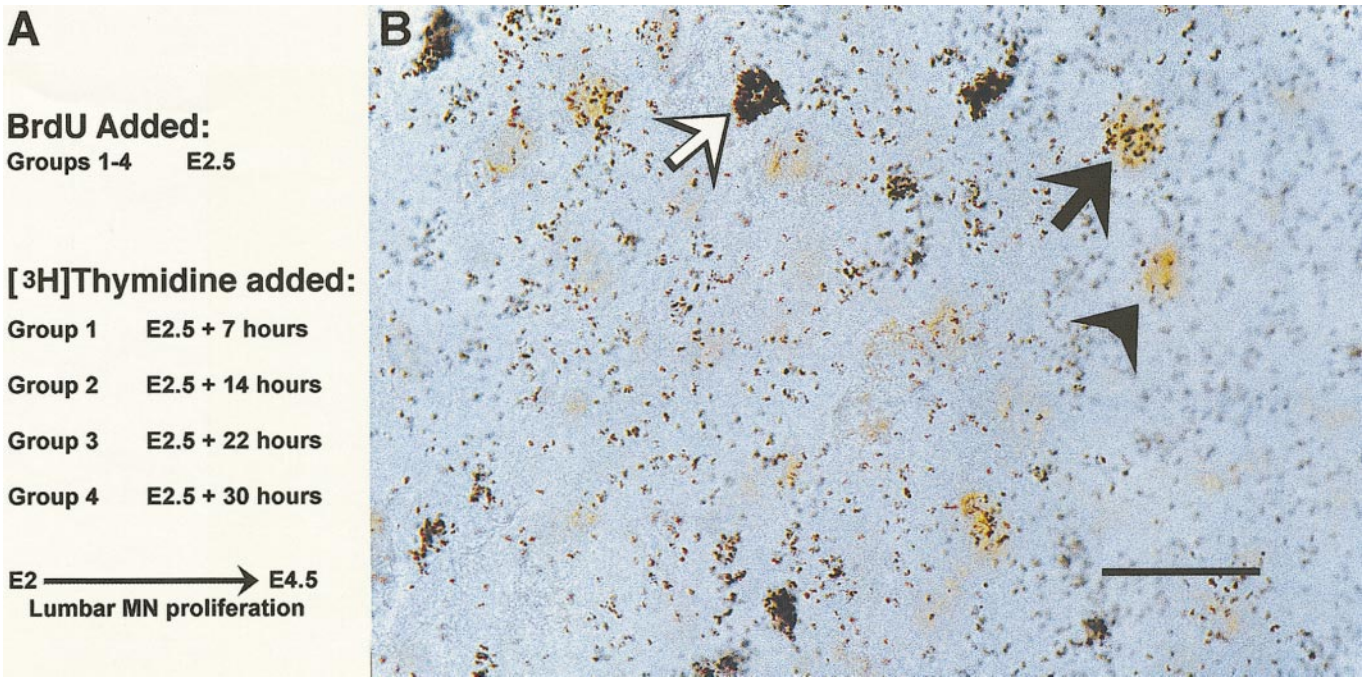


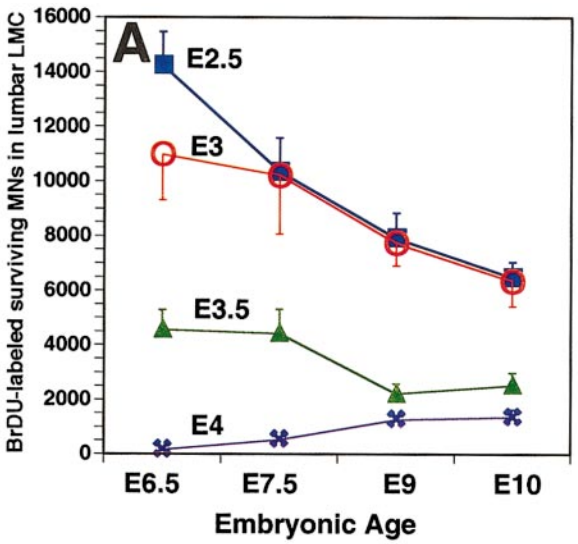
FIG. 2. BrdU is available for uptake by proliferating MNs for at least 14 h. (A) Schematic representation of the experiment in which BrdU is added at E2.5 and [^{3}H]thymidine is added at specific times afterward during MN proliferation. Because a mitotically active cell can incorporate BrdU and [^{3}H]thymidine if they are both available, the interval between the delivery of these two S-phase markers that results in zero double-labeled MNs indicates the time at which BrdU is no longer available for uptake by proliferative MNs. (B) Transverse section of the lumbar LMC at E13 from an embryo which received BrdU at E2.5 and [^{3}H]thymidine 14 h later. At this interval, several BrdU-labeled, [^{3}H]thymidine-labeled MNs can be seen (dark arrow) in addition to BrdU-labeled MNs (arrowhead) and [^{3}H]thymidine-labeled MNs (white arrow). Scale bar, 15 μm.

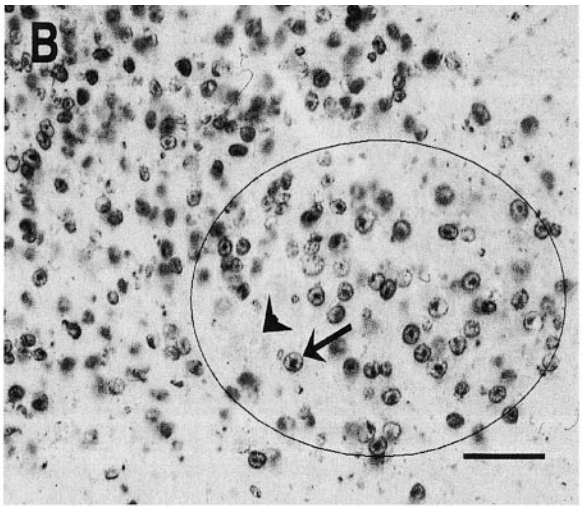
[^{3}H]BrdU, when administered at a dose of 20 μg/egg on E3, remains available for 8 h (Bannigan *et al.*, 1981; Bannigan, 1987). We tested the 2.5 μg/egg dose given at E2.5 by adding [^{3}H]thymidine at specific time points after BrdU, reasoning that the interval of time which results in no [^{3}H]thymidine/BrdU-colabeled MNs reflects the time for uptake of BrdU (Fig. 2A). Seven hours after treatment with BrdU, delivery of [^{3}H]thymidine resulted in significantly more [^{3}H]thymidine/BrdU-colabeled MNs compared to [^{3}H]thymidine administration after 14, 22, or 30 h after BrdU (Table 1). Figure 2B shows the lumbar LMC of an E13 embryo given [^{3}H]thymidine 14 h after E2.5 treatment with BrdU. These data demonstrate that the availability of BrdU for uptake by DNA-synthesizing MNs is mainly restricted to the first 12–14 h after delivery, although a few MNs incorporate both labels even when their delivery is separated by 14 h.

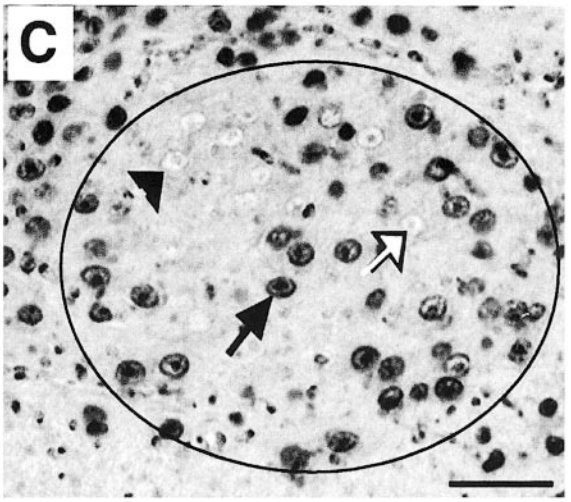
The Temporal Pattern of Lumbar MN Death Reflects Their Time of Birth

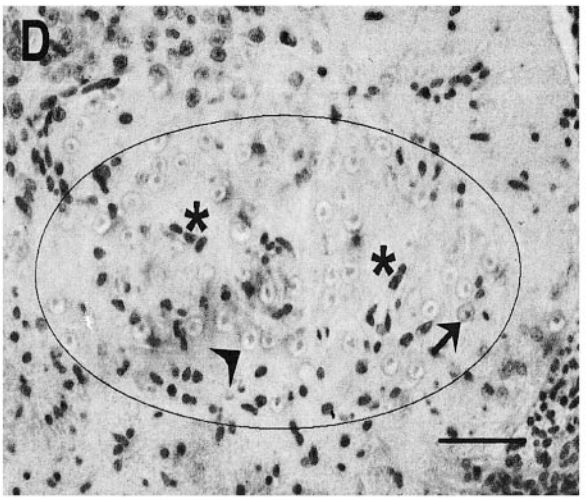
Because avian embryonic MNs begin to die within the lumbar LMC at E6.5, exhibiting peak cell death at E7.5, and PCD continues until about E10–E11 (Hamburger, 1975), we sought to determine if this temporal gradient in MN PCD

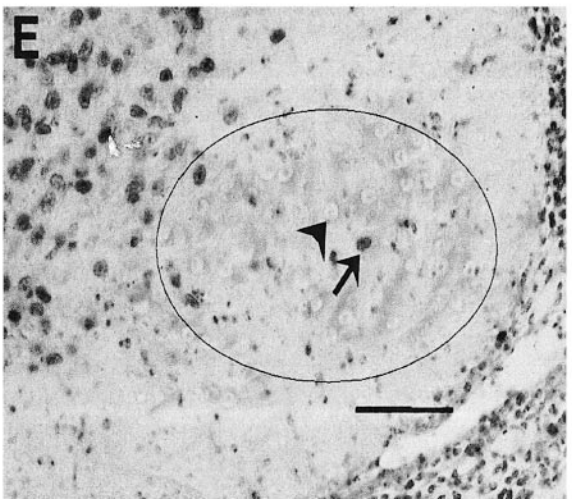
FIG. 3. (A) The number (mean ± SD) of BrdU-labeled MNs in the lumbar LMC during the period of PCD as a function of the time of administration of BrdU. More MNs are labeled when BrdU is given at E2.5 or E3 (squares and circles, respectively) than at E3.5 or E4 (triangles and crosses, respectively). BrdU-labeled MNs born before E4 are reduced during PCD. (B–F) Transverse sections of the lumbar LMC (in outline) at E9 in chick embryos given BrdU at E2.5 (B), E3 (C), E3.5 (D), E4 (E), or E5 (F). Most unlabeled MNs (arrowheads in B–E) born before E2.5 or E3.0 (B, C) are located medially in the LMC, though some can be found in more lateral regions of the LMC (white arrow in C). BrdU-labeled MNs (black arrows in B–E) are found increasingly laterally (B–D) in the LMC, but not all MNs born after E4 are located laterally (black arrow in E). Administration of BrdU at E3.5 results in the labeling of glia within the LMC (asterisks in D). Delivery of BrdU on E5, after MN proliferation, results in the absence of BrdU-labeled MNs within the LMC (F). Scale bar in B–F, 50 μm.











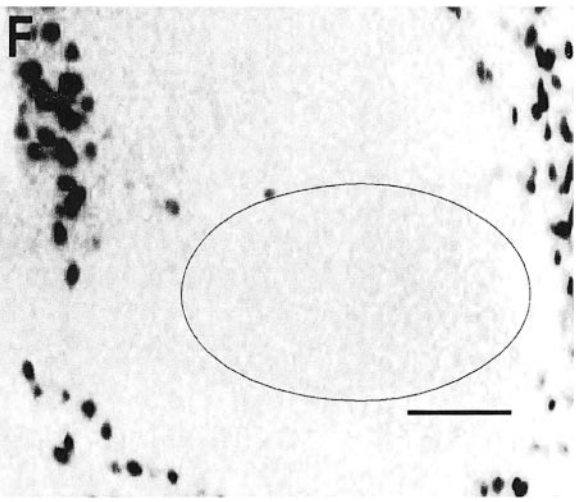


TABLE 2

The Relative Contribution to MN PCD by Subpopulations of MNs Born within Different Periods of MN Neurogenesis[a]

	Pyknotic MNs during MN PCD ± SD				
Day given BrdU	E6.5	E7.5	E9	E10	%
E2.5	260 ± 40	120 ± 23	24 ± 17	32 ± 41	41
E3	136 ± 26	270 ± 62	48 ± 36	12 ± 11	45
E3.5	16 ± 17	24 ± 22	40 ± 20	4 ± 9	8
E4	0 ± 0	4 ± 9	16 ± 17	40 ± 20	6

[a] For each subgroup, the number of pyknotic BrdU-labeled MNs (mean ± SD) at four different time points during PCD was summed. The contribution to the total amount of pyknotic BrdU-labeled MNs during PCD by MNs born in each 12-h period is presented in the last column as a percentage ($n = 5$).

could be attributed to subpopulations of MNs born at increasingly later times. The temporal pattern of cell death was first investigated by counting healthy BrdU-labeled MNs at various times during MN PCD after BrdU delivery at specific time points during MN genesis. Figure 3A shows that the administration of BrdU at E2.5 and E3 results in the highest number of healthy BrdU-immunolabeled neurons and that PCD reduces these numbers. Figures 3B–3E show BrdU-immunopositive sections of the lumbar LMC at E9 after BrdU was given at E2.5, E3, E3.5, and E4, respectively. Most unlabeled MNs born between E2 and E2.5 can be seen medially in Figs. 3B and 3C, consistent with previous reports that the medial LMC is composed of earlier born MNs and that later born MNs settle laterally (Hollyday and Hamburger, 1977). In Fig. 3D, many apparent labeled glia can be visualized in the LMC in embryos given BrdU at E3.5. In embryos given BrdU at E4, only a few BrdU-positive healthy MNs are seen in each section, consistent with the observation that 90% of lumbar MN genesis is complete by this time (Hollyday and Hamburger, 1977). Unexpectedly, however, not all of these late-born lumbar MNs were found at the lateral edge of the LMC (Fig. 3E; see Discussion). Finally, transverse sections of the LMC of an E9 embryo receiving BrdU at E5, after the end of MN proliferation, show an absence of BrdU-labeled MNs, as expected (Fig. 3F).

In order to determine whether a temporal relationship exists between MN birth date and PCD, we next analyzed the relative contribution to the overall amount of dying MNs by subgroups of MNs born at specific times during MN neurogenesis (Table 2). MNs born between E2.5 and E3.5 account for ~85% of dying MNs during PCD, indicating that the majority of MNs undergoing PCD are generated during the period of highest MN proliferation (Hollyday and Hamburger, 1977). In addition to the finding that MNs born at all time points during their genesis contribute to MN PCD (Table 2), these data suggest that the time period during which MNs are born influences the timing and extent of cell death. This hypothesis was tested by expressing the number of dying MNs within each BrdU-labeled subpopulation as a percentage of 1000 surviving MNs within that population. Figures 4A–4D show that the majority of MNs generated between E2.5 and E3.5 that die do so in the first half of the MN PCD period and that MNs born after E3.5 die almost exclusively within the latter half of MN PCD. Specifically, more MNs born between E2.5 and E3 die at E6.5 ($P < 0.005$), more MNs born between E3 and E3.5 die at E7.5 ($P < 0.005$), more MNs born between E3.5 and E4 die at E9 ($P < 0.005$), and more MNs born after E4 exhibit cell death at E10 or later. As the numbers of dying MNs born after E4 are relatively small, a small difference in their number between embryos resulted in a large standard deviation.

The Rostrocaudal but Not Mediolateral Pattern of Dying MNs Reflects Their Birth Date

Because earlier findings reported a rostrocaudal gradient of dying lumbar MNs during PCD (Hamburger, 1975; Williams *et al.*, 1987), we asked whether MN birth date could account for this pattern. We first analyzed the rostrocaudal distribution of healthy MNs at the onset of PCD, E6.5. The numbers of healthy BrdU-labeled MNs in rostral and caudal halves of the lumbar LMC were similar (Table 3A), indicating that the rostrocaudal settling pattern of MNs before PCD does not restrict the pattern of death to particular rostrocaudal locations. We then analyzed the rostrocaudal distribution of dying BrdU-labeled MNs throughout the period of PCD (Table 3B). Dying MNs born between E2.5 and E3.5 are found significantly in the rostral half of the lumbar spinal cord at E6.5 and E7.5, dying MNs born after E4 are located significantly in the caudal half at E9 and E10, and dying MNs born between E3.5 and E4 do not appear to be localized preferentially to any rostrocaudal region of the lumbar spinal cord.

Finally, because MNs born earliest reside within medial portions of the LMC and those born later occupy lateral regions of the LMC, and because medial LMC MNs project to ventrally derived muscles and lateral LMC MNs to dorsally derived muscles (Hollyday and Hamburger, 1977; Landmesser, 1978b), we examined the mediolateral gradient of dying MNs born at different times. In order to analyze this pattern of cell death among all lumbar MNs, we divided the LMC into medial and lateral halves and counted dying MNs

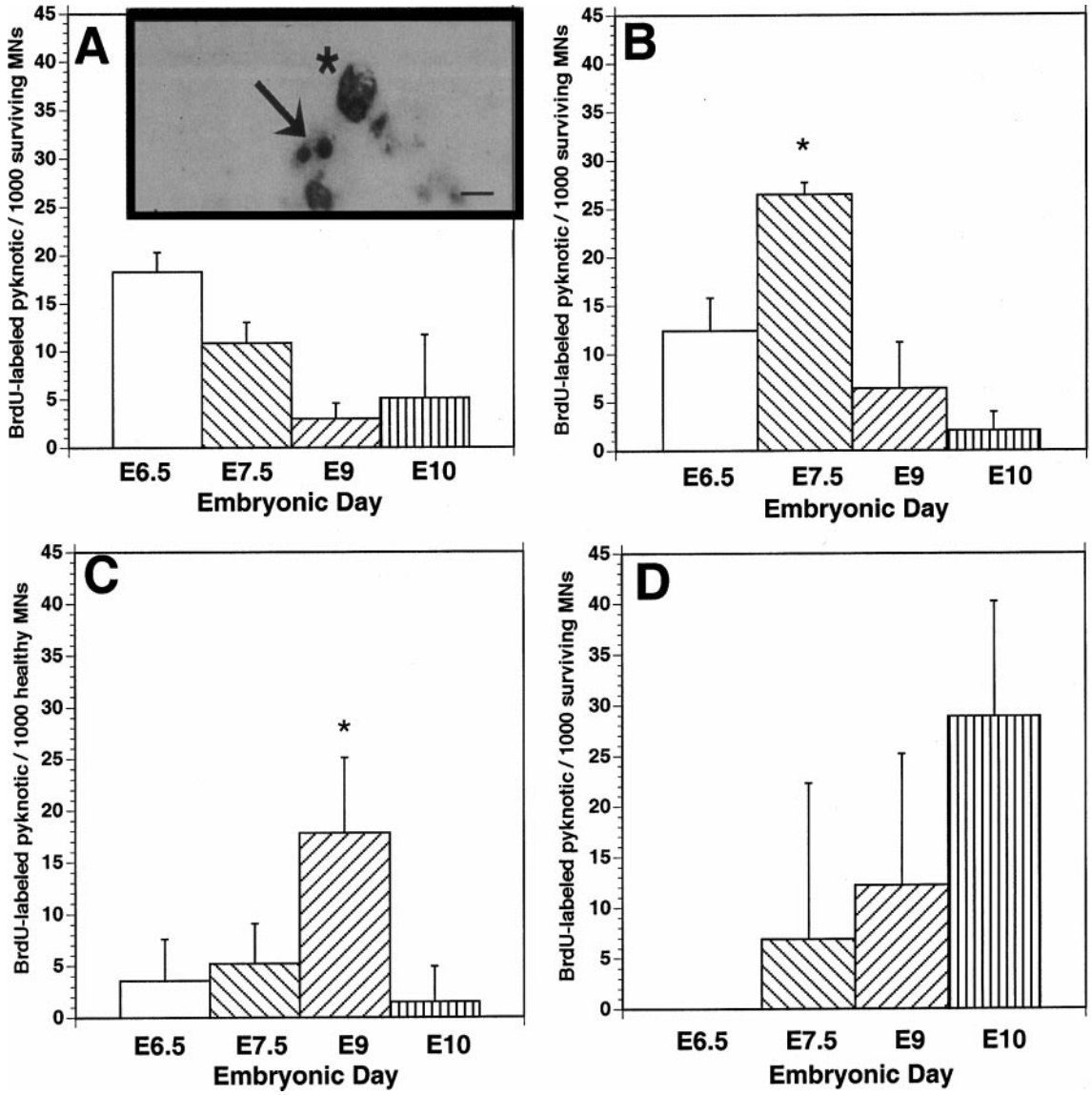


FIG. 4. The temporal relationship between lumbar MN birth and death. Of the four time points during PCD at which dying BrdU-labeled MNs were recorded, embryos given BrdU at E2.5 exhibit the most death at E6.5 (A), those given BrdU at E3.0 display the most at E7.5 (B), those given BrdU at E3.5 exhibit the most at E9 (C), and finally those given BrdU at E4.0 display the most death at E10 (D). Values represent the mean percentages (± SD) of BrdU-labeled pyknotic MNs/per 1000 BrdU-labeled healthy MNs ($n = 5$ embryos per group). *$P < 0.005$ vs other ages in *t* tests with a Bonferroni correction. The inset in A shows a BrdU-labeled pyknotic MN (arrow) and a BrdU-labeled healthy MN (*) which would not have been included in cell counts because its nuclear membrane is not clearly in the plane of section. Scale bar, 10 μm.

first within thionin-stained sections of the spinal cord during PCD. Table 4A shows that the location of dying MNs is mostly medial at E6.5 but spreads laterally during PCD. Table 4B presents the same spatial analysis of dying MNs but also presents these data on the basis of MN birth date. A statistically significant number of MNs born between E2.5 and E3 that die from E6.5 to E7.5 are found medially within the lumbar LMC. Similarly, MNs which are born between E3 and E3.5 and die between E7.5 and E9 tend to be located in lateral positions of the LMC. Other populations of dying MNs defined by birth date do not appear to reside within specific mediolateral locations within the LMC.

DISCUSSION

These experiments provide the first *in vivo* demonstration that the temporally staggered pattern of MN PCD

TABLE 3
The Rostrocaudal Gradient of Dying MNs in the Lumbar LMC[a]

(A) Healthy BrdU-labeled MNs in rostral and caudal halves at the onset of PCD

Day given BrdU	Rostrocaudal half of LMC	BrdU-labeled healthy MNs at E6.5
E2.5	Rostral	3600 ± 525
	Caudal	3660 ± 430
E3.0	Rostral	2740 ± 560
	Caudal	2840 ± 400
E3.5	Rostral	1100 ± 270
	Caudal	1270 ± 380
E4	Rostral	80 ± 30
	Caudal	110 ± 40

(B) Dying BrdU-labeled MNs in rostral and caudal halves at four time points during PCD

Day given BrdU	Rostrocaudal half of LMC	BrdU-labeled pyknotic/1000 healthy MNs during PCD			
		E6.5	E7.5	E9	E10
E2.5	Rostral	10.8 ± 2.4*	8.6 ± 2.8*	2.1 ± 1.5*	0.9 ± 1.2
	Caudal	6.9 ± 1.5	2.5 ± 1.9	0 ± 0	2.5 ± 3.6
E3	Rostral	7.6 ± 2.9*	17.4 ± 3.0*	1.8 ± 2.7	0.4 ± 0.7
	Caudal	4.9 ± 0.5	10.9 ± 3.3	3.7 ± 3.3	0.9 ± 1.1
E3.5	Rostral	3.0 ± 2.8	3.3 ± 3.0	10.3 ± 7.3	0 ± 0
	Caudal	0.7 ± 1.5	2.3 ± 3.5	8.4 ± 3.1	1.6 ± 3.7
E4	Rostral	0 ± 0	3.0 ± 2.2	1.5 ± 1.8	0 ± 0
	Caudal	0 ± 0	3.0 ± 1.0	9.4 ± 3.3*	26.2 ± 9.5*

[a] The lumbosacral spinal cord was divided into rostral and caudal halves and the number of healthy (A) or dying (B) BrdU-labeled MNs was counted from different-born subpopulations at the onset of PCD on E6.5 (A) or at four time points during PCD (B). (A) Values represent the numbers (mean ± SD) of healthy BrdU-labeled MNs at E6.5 ($n = 5$). (B) Values represent the numbers (mean ± SD) of BrdU-labeled pyknotic MNs per 1000 BrdU-labeled healthy MNs in either the rostral or the caudal half of the lumbar LMC at E6.5, E7.5, E9, and E10 ($n = 5$).

* $P < 0.05$, rostral vs. caudal, Student t test.

reflects the same pattern of MN birth several days earlier. These data complement *in vitro* studies showing that MN cell cycle exit and acquisition of trophic factor dependence are temporally correlated phenomena (Mettling *et al.*, 1993, 1995). Furthermore, the rostrocaudal gradient in MN proliferation and differentiation, at least within the lumbar region of the spinal cord, is recapitulated by the same spatial gradient of MN PCD. These data suggest that MNs are endowed with intrinsic properties regulating their survival during PCD which vary among subpopulations of MNs born at different times. Further experiments are needed to substantiate this (e.g., do these properties change or remain the same if the lumbar spinal cord is transplanted to brachial or thoracic segments?) and to examine the cellular/molecular basis of the putative intrinsic signals.

The observation that MNs born between E2.5 and E3.5 die mostly 3–5 days later and those born between E3.5 and E4.5 die mostly 5–6 days later (Fig. 4) implies that although these subsets of MNs die in a temporal progression consistent with their time of birth, they do not all die at a stereotypic time after they exit the cycle, e.g., at the expiration of a fixed, intrinsic clock. This may reflect the possibility that MNs with different birth dates remain independent of trophic support for a period of time which varies depending upon the distance their axons must traverse to reach their targets, as has been found in several populations of cranial ganglia sensory neurons (Vogel and Davies, 1991). This hypothesis has been difficult to assess *in vivo* within pools of hindlimb-innervating MNs, whose axons form the lumbar spinal nerves, all of which appear to project to the base of the limb bud at about the same time prior to limb innervation (Fouvet, 1973; Tosney and Landmesser, 1985). Evidence against the idea that target muscle distance regulates the period of MN trophic dependence and therefore survival during PCD, however, is the finding that leg musculature at different proximodistal locations becomes functionally innervated (at stages 27–28) at about the same time (Landmesser and Morris, 1975). Once the distance between different subgroups of lumbar MNs and their targets (which exist only as premuscle masses at the initial

TABLE 4
Analysis of the Mediolateral Pattern of Dying MNs in the LMC[a]

(A) Thionin-stained sections

Mediolateral half of LMC	Pyknotic MNs during PCD			
	E6.5	E7.5	E9	E10
Medial	270 ± 70*	330 ± 35	70 ± 13	43 ± 17
Lateral	15 ± 10	333 ± 21	100 ± 10*	63 ± 20

(B) BrdU-immunolabeled sections

Day given BrdU	Mediolateral half of LMC	Pyknotic BrdU-labeled MNs during PCD			
		E6.5	E7.5	E9	E10
E2.5	Medial	205 ± 12*	65 ± 9*	8 ± 13	17 ± 4
	Lateral	50 ± 5	31 ± 6	10 ± 5	23 ± 6
E3	Medial	62 ± 17	72 ± 16	0 ± 0	6 ± 6
	Lateral	44 ± 15	201 ± 13*	36 ± 8*	10 ± 5
E3.5	Medial	9 ± 8	16 ± 4	20 ± 12	—
	Lateral	9 ± 8	19 ± 7	20 ± 9	—
E4	Medial	—	—	5 ± 5	19 ± 8
	Lateral	—	—	5 ± 3	18 ± 6

[a] (A) The pattern in thionin-stained transverse sections of embryos at E6.5, E7.5, E9, and E10, which therefore represents MNs from the entire proliferative period. (B) The same pattern at the same four time points during PCD, which represents that subpopulation of MNs born in the specified period. Values represent the numbers (mean ± SD) of dying thionin-stained (A) or BrdU-labeled (B) MNs in the medial and lateral halves of the lumbar LMC ($n = 3$). Dashes signify those time points at which fewer than 10 pyknotic BrdU-labeled MNs were detected.

* $P < 0.05$, medial vs lateral, Student t test.

time of innervation) is known, it may then be possible to determine the relationship, if any, between birth date and target distance (and therefore survival) by administering both systemic BrdU and a retrograde tracer to muscles located at different distances. In this way, the times of birth and death and the distance between MNs and their target muscle could be assessed.

We have identified subpopulations of MNs based on their time of birth by delivering BrdU at specific intervals during MN neurogenesis. The accuracy of this technique for selectively labeling one population of dividing MNs was addressed by determining the period of time after delivery that BrdU was available for incorporation by these cells. The fact that some MNs are included in more than one of the four subpopulations defined by their birth date is supported by both the fact that some BrdU/[^{3}H]thymidine-colabeled MNs are found after 12 h (at the 14-h interval) and by the fact that the summation of the number of healthy BrdU-labeled MNs in the four groups at any time point during PCD results in MN numbers that exceed the published values for that time point by ~20% (Williams *et al.*, 1987). This overestimation may also occur partly as a result of counting the progeny of earlier BrdU-labeled MNs. These later born, weakly BrdU-labeled MNs may appear to be proliferative during an earlier time interval than they actually were and may therefore account for the overestimation of the number of dividing MNs. Despite such caveats, these data nonetheless clearly indicate a temporal pattern of PCD among lumbar MNs which mainly reflects the corresponding 12-h period during which they became postmitotic.

BrdU administration at various intervals during spinal MN proliferation resulted in the labeling of MNs whose mediolateral settling pattern within the lumbar LMC resembled that of previous studies (Hollyday and Hamburger, 1977). MNs in one population which failed to observe this pattern were those born after E4, which settled throughout, rather than solely at the lateral extremes of, the LMC (Fig. 3E). These MNs appear to be significantly different from earlier born MNs, as their numbers increase between E6.5 and E10, decrease due to cell death after E10 (Fig. 3D and data not shown), and acquire trophic dependence significantly later than earlier born neurons (Mettling *et al.*, 1995). The increased number of these MNs which we observed between E6.5 and E10 may reflect a later period of migration for MNs born after E4. This increase, which represents 5–10% of all lumbar spinal MNs, may normally be obscured because of the overall decrease in MN number owing to the PCD of earlier born MNs, which account for 90–95% of all lumbar spinal MNs.

In addition to dying along a temporal gradient of progres-

sion, subgroups of lumbar MNs also die along a spatial gradient. Because MNs are generated along a slight rostrocaudal gradient within both the brachial and the lumbar LMC (Hollyday and Hamburger, 1977), we first compared the numbers of healthy labeled MNs in rostral and caudal halves before the period of PCD. Surprisingly, we found no such difference for MNs born in any 12-h interval (Table 3A). This result may reflect the fact that this gradient is modest; for example, ~60% of the MNs born before E3.5 are located in the rostral half at E10 vs ~40% in the caudal half; MNs born after this time are located equally in either half (Hollyday and Hamburger, 1977). These data show that the rostrocaudal location of a dying MN during PCD is not restricted to either half of the lumbar LMC based on its time of generation; MNs born throughout the period of proliferation settle in both rostral and caudal halves of this region of the spinal cord.

Dying MNs born before E3.5 are observed more frequently in the rostral than in the caudal half of the lumbosacral region, especially at E6.5 and E7.5, those time points during PCD at which the peak of cell death is occurring for these neurons. Dying MNs born between E3.5 and E4 are located throughout all rostrocaudal levels, and dying MNs born after E4 are found predominantly within the caudal half at E9 and E10 (Table 3B). These data are consistent with the observation that the overall pattern of dying MNs within the lumbar spinal cord appears to progress rostrocaudally (Hamburger, 1975; Williams *et al.*, 1987). Such a pattern of cell death is also consistent with the finding that mice lacking the *hoxc-8* gene display an altered rostrocaudal pattern of MNs innervating the distal forepaw (Tiret *et al.*, 1998). MNs which normally would not supply these muscles because of their rostrocaudal identity lose this restriction in the mutant embryos and therefore compete with the appropriate MNs in forming these connections, resulting in an aberrant rostrocaudal pattern of cell death.

In addition, the spatial pattern of MNs undergoing PCD also progresses in a mediolateral gradient over time (Table 4A), which appears to reflect the same gradient in the settling pattern of postmitotic MNs (Hollyday and Hamburger, 1977). The mediolateral position at which some but not all MNs die during PCD is influenced by their birth date (Table 4B), consistent with the observation that each stage during proliferation includes MNs which supply both ventrally and dorsally derived muscles (Hollyday, 1980a).

The fact that mice deficient in LIF, CNTF, or GDNF receptors display only a moderate increase in the loss of MNs (DeChiara *et al.*, 1995; Li *et al.*, 1995; Cacalano *et al.*, 1998) suggests that specific MNs may be capable of responding to selective trophic factors. This hypothesis is also supported by the observation that addition of specific trophic factors rescues limited numbers of MNs destined to undergo PCD (Oppenheim, 1996) and by the increasing number of reports of selective trophic factor receptor expression in subgroups of MNs (Yamamoto *et al.*, 1997; Novak *et al.*, 1999). Together with the results presented here, these data suggest that MNs which are born at specific times may express selective trophic factor receptors, innervate specific targets, and undergo PCD at prescribed locations and times. For example, in the subpopulation of MNs which are born between E2.5 and E3 and which die at E6.5, 70% are found at a specific rostral–medial position in the lumbar LMC. This location coincides with the adductor motor pool and expression of the LIM- and ETS-family transcription factors Isl1, Isl2, and ER81 (Landmesser, 1978a; Lin *et al.*, 1998). This suggests that the settling position within the spinal cord is one important component of the process by which the birth date of spinal MNs influences their timing of PCD. Studies are in progress to determine whether MN subtypes based on birth date, position, ETS-factor expression, or peripheral targets express distinct patterns of trophic factor receptors and require different target-derived trophic factors for their survival.

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